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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EU 588457825 US

INVENTOR(S)		
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☑ *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Assembly of Chitosan onto an Electrode Surface

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 17
☑ Drawing(s) *Number of Sheets* 6
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☑ Other (specify) Parts listed in cover letter

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fees
☑ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 500770
☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

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Date 08/23/2002

REGISTRATION NO. *(if appropriate)* 51,269

Docket Number: Payne02-019-PRV

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

PROVISIONAL APPLICATION COVER SHEET
Additional Page

PTO/SB/16 (02-01)
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Docket Number	Payne02-019-PRV

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Number 1 of 1

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Box Provisional Application

Re: U.S. Provisional Patent Application under 37 C.F.R. § 1.53 (c)
Appl. No. *To Be Assigned*; Filed: *Herewith*

For: **ASSEMBLY OF CHITOSAN ONTO AN ELECTRODE SURFACE**
Inventors: Wu et. al.
Our Ref: Payne 02-019 PRV

Sir:

The following documents are forwarded herewith for appropriate action by the U.S. Patent and Trademark Office:

1. USPTO Provisional Application for Patent Cover Sheet PTO/SB/16;

2. U.S. Provisional Patent Application entitled:

ASSEMBLY OF CHITOSAN ONTO AN ELECTRODE SURFACE

and naming as inventors:

Li-Qun Wu
Anand P. Gadre
Mark J. Kastantin
Hyunmin Yi
Gary W. Rubloff

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Gregory F. Payne

the application comprising:

a. specification containing:

i. 15 pages of description prior to the claims;

ii. 1 pages of claims (11 claims);

iii. a one (1) page abstract;

b. 6 sheets of drawings: (Figures 1-6);

c. 0 pages of sequence listing;

3. One (1) return prepaid postcard.

The name of the assignee is University of Maryland Biotechnology Institute.

Correspondence should be sent to Customer Number 32417.

It is respectfully requested that the attached postcard be stamped with the filing date and unofficial application number and returned as soon as possible.

[redacted]

The Applicants claim small entity status under 37 C.F.R. § 1.27.

The Commissioner is hereby authorized to charge $80.00 to Deposit Account Number: 500770 (provisional application filling fee of $80.00)

Respectfully submitted,

University of Maryland
Biotechnology Institute

Peter P. Tung
Agent for Applicants
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ASSEMBLY OF CHITOSAN ONTO AN ELECTRODE SURFACE

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William E. Bentley
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Gregory F. Payne

The government may have certain rights to this invention, pursuant to Grant No. BES-0114790, awarded by the National Science Foundation.

Background of the Invention

The ability to create devices (e.g. biosensors, microarrays, and microelectromechanical systems (MEMS)) requires facile methods to precisely control surfaces. A variety of patterning techniques can be used to produce desired structures, while various methods have been investigated to control surface chemistries. For instance, surface chemistries have been controlled by self-assembling monolayers using reactions between thiols and metal surfaces,[1,2] or between alkyltrichlorosilanes and oxidized silicon.[3-5] An additional method to assemble macromolecules and particles is to exploit an applied voltage.[6] Applied voltages have been used to assemble colloidal particles,[7] proteins,[8-10] and cells[11] onto electrode surfaces.

Chitosan is an amine-rich polysaccharide derived by deacetylation of chitin. Chitin is the second most abundant polysaccharide in nature and is found in crustaceans, insects, and fungi. Chitosan is becoming an increasingly important biopolymer because it offers unique physicochemical properties.[12] Specifically, chitosan has primary amino groups that have pKa values of about 6.3.[13,14] At pHs below the pKa, most of the amino groups are protonated making chitosan a water-soluble, cationic polyelectrolyte. Chitosan's water-solubility is unique as other β,(1→4)-linked polysaccharides (e.g. cellulose and chitin) are insoluble. At pHs above the pKa, chitosan's amino groups are deprotonated, and this polymer becomes insoluble. Chitosan's pH-dependent solubility is attractive because it allows processing from aqueous solutions[15] while a modest increase in pH to neutrality enables chitosan to be formed into various shapes (e.g. beads, membranes, and films). An additional feature is that chitosan's amino groups confer nucleophilic properties to this polymer. Specifically, the deprotonated amino groups have an unshared electron pair that can undergo reaction with a variety of electrophiles. As a result, various chemistries can be exploited to crosslink chitosan and to graft substituents onto this polymer.[16-26]

Brief Summary of the Invention

We disclose herein a method of deposition of the polysaccharide chitosan onto the surface of an electrode substrate and substrates comprising a thin layer of bound chitosan. Chitosan was deposited onto the surface of an electrode in response to an applied voltage. Electrodes were contacted with a chitosan solution, with a thin layer of chitosan deposited on the surface of the electrode.

Brief Description of the Several Views of the Drawings

Figure 1. Diagram of chitosan deposition.

Figure 2. Deposition from chitosan solution onto the surface of an electrode. Deposition occurred from a 1 w/v% chitosan solution using an applied voltage of 2.5 V. As discussed in the text some electrodes were measured after being neutralized with 1M NaOH.

Figure 3. SEM micrograph of deposited layer on an electrode (a) without neutralization and (b) with neutralization. See text for details.

Figure 4. Deposition under varying conditions. (a) Deposition occurred from a 1 w/v% chitosan solution using an applied voltage of 2.5 V. (b) Deposition was measured after 6 minutes using chitosan solutions of varying concentrations and an applied voltage of 2.5 V. (c) Deposition was measured after 6 minutes from a 1 w/v%

chitosan solution using varying voltages. In all cases, the electrode was immersed in caustic, rinsed extensively, and dried prior to measuring thickness.

Figure 5. IR spectrum of deposited material and chitosan. Material deposited on the electrode was neutralized in base, extensively washed with distilled water, and dried. The IR spectrum was collected using a KBr pellet. The control spectrum was collected using a chitosan film.

Figure 6. ES-MS spectrum of deposited material after incubation with chitosanase. See text and Table 1 for details.

Detailed Description of the Invention

As defined herein, a substrate of the instant invention is any compound which can function as an electrode whereby chitosan is deposited onto the substrate according to Figure 1 or as disclosed in the instant specification. Suitable substrates for chitosan deposition would include any electrically conducting compounds including but not limited to metals such as aluminum, antimony, cadmium, chromium, cobalt, copper, gold, iron, lead, magnesium, mercury, nickel, palladium, platinum, silver, steel, tin, tungsten, zinc, and alloys thereof. As defined herein, a cell may be eucaryotic or prokaryotic and may be from any source where cells can be obtained. For the chitosan solution used to deposit chitosan onto a substrate, suitable concentrations of chitosan vary from 0.0001 to 0.001 (w/v) %, 0.001 to 0.01 (w/v) %, 0.01 to 0.1 (w/v) %, 0.1 to 1 (w/v) %, 1 to 10 (w/v) %, 10 to 20 (w/v), and 20 to 30 (w/v) %. Suitable pH for deposition of chitosan onto a substrate is any pH where chitosan remains soluble and in

solution. It is further recognized that the concentration of the chitosan solution, voltage and the time a current is applied to deposit chitosan onto a substrate can be varied to control the extent of chitosan deposition.

A preferred embodiment of the instant invention is method of depositing chitosan onto a metal substrate comprising the steps of : a) contacting said substrate with a chitosan solution; and b) applying an electric current to the substrate, wherein the substrate is in a circuit with an electrode also in contact with said chitosan solution. A further preferred embodiment of the method of depositing chitosan onto a metal substrate further comprises washing the substrate containing deposited chitosan with at least one liquid selected from the group consisting of water, a solution with neutral pH, a basic solution and an acidic solution. Another preferred embodiment of the method of depositing chitosan onto a metal substrate further comprises contacting the substrate with bound chitosan with chitosanase.

A preferred embodiment of the instant invention is a substrate coated with chitosan. A further preferred embodiment is a substrate coated with chitosan further comprising bound protein. Another preferred embodiment is a substrate coated with chitosan further comprising a bound enzyme. Another preferred embodiment is a substrate coated with chitosan further comprising bound polynucleotide. Yet another preferred embodiment is a substrate coated with chitosan further comprising either bound RNA or DNA. Still another preferred embodiment is a substrate coated with chitosan further comprising bound cells. A further preferred embodiment of the inventions is a substrate coated with chitosan wherein the substrate is a metal.

Example 1

MATERIALS AND METHODS

Chitosan from crab shells (85 % deacetylation as reported by the supplier) and the enzyme chitosanase were purchased from Sigma-Aldrich Chemicals. Chitosanase was reported by the manufacturer to have specific activities of 102.3 U/mg. Chitosan solutions were prepared by adding chitosan flakes to water and incrementally adding small amounts of HCl to the solution to maintain the pH near 3. After filtering undissolved material, these chitosan solutions were diluted to various concentrations, and the pH was adjusted to 5.0 using NaOH (1 M).

Electrodes were prepared by depositing 90 Å thick chromium (Cr) and then 2000 Å thick gold (Au) films on 4-inch diameter silicon wafers already coated with 1-micron thick thermal oxide film. For chitosan deposition, the electrodes were dipped into a chitosan solution (pH=5, 1% w/v) as shown in Scheme 1. In most experiments, three electrodes were examined. Two of the electrodes (positive and negative) were connected to a DC voltage supply using alligator clips. The third electrode was not connected to a power supply and is designated a "neutral" electrode. At specific times the electrodes were removed from the solution and rinsed with distilled water, after which the voltage was removed. In some cases, electrodes were immediately oven-dried (60 °C for 3 hours). In other cases, electrodes were neutralized by immersion in a basic solution (1 M NaOH) and then rinsed with distilled water prior to drying. After drying, the thickness of the deposited layers were measured by a profilometer (ALPHA-STEP 500 SURFACE PROFILER, TENCOR Instruments). Thicknesses were measured on different areas of the electrode surface and the average values were calculated.

Scanning electron microscopy (SEM) was used to study the surface morphology of the deposited layer. SEM micrographs have been recorded using a Focused Ion Beam system (FIB/SEM workstation dual beam 620; FEI Company). Samples on silicon substrates were placed in the chamber having vacuum of about 10^{-6} Torr. Structural properties were examined at a 20,000-fold magnification.

For chemical analysis, deposition was obtained by placing electrodes in a chitosan bath (1 w/v %; pH = 5) for 20 minutes with an applied voltage of 2.0 volts. For IR analysis, the negative electrode was removed from the chitosan solution, rinsed, disconnected from the power supply, and then placed in 1 M NaOH overnight. When the electrode was soaked in base for such a long time, the deposited material was observed to detach from the electrode surface. This deposited material was then extensively washed with distilled water and dried overnight at 60 °C. After drying, it was ground with KBr powder and pressed into a pellet. IR spectra were collected using a Perkin-Elmer 2000 FT-IR system.

For analysis by electrospray mass spectrometry (ES-MS), the negative electrode was removed from the chitosan solution, rinsed, disconnected from the power supply, and then placed in a small volume of dilute acid (HCl; pH=3) and held overnight to allow the deposited material to dissolve. This acid solution was recovered, diluted to approximately 0.08 w/v % and the pH was adjusted to 5.5. The sample was then incubated for one day at 37 °C with the enzyme chitosanase (0.2 U/ml). After incubation the solution was filtered to remove precipitates, and analyzed by ES-MS (Thermo Finnigan, San Jose, CA, USA). All samples for ES-MS analysis were diluted in an aqueous solution containing 0.1 % formic acid and analyzed in positive ion mode.[27]

RESULTS AND DISCUSSION

To examine deposition, we immersed electrodes in an acidic chitosan solution and applied a voltage of 2.5 V. After applying the voltage for varying times, negative electrodes were removed from the solution, rinsed with distilled water, and the voltage was removed. In some cases, the electrodes were dried, while in other cases they were immersed in base, rinsed and then dried. After drying, the thickness of the deposited layer was measured by profilometry. Figure 1 shows that the thickness of the deposited layer increases over time. Additionally, Figure 1 shows that the thickness of the deposited layer is less when the electrode was treated with base.

To examine the surface morphology of the negative electrodes we used scanning electron microscopy (SEM). Figure 2a shows micrographs for electrodes that were dried without neutralization. As can be seen from Figure 2a, this sample has a non-uniform surface morphology. Possibly, the surface roughness of this electrode may be due to the presence of salts associated with the chitosan polyelectrolyte. Figure 2b shows the surface of a negative electrode that had been immersed in base and rinsed extensively before drying. As indicated in Figure 2b, the surface of this electrode is more uniform – presumably due to the neutralization of chitosan. The observation in Figure 1 that deposited layers are thinner after neutralization suggests that neutralization leads to a collapse of the polymer network and possibly also the elimination of salts. In subsequent experiments, neutralization was performed prior to measuring the thickness of deposited layers.

Additional studies were performed to characterize deposition, and to compare deposition onto the negative and positive electrodes. Figure 3a shows that the thickness of the deposited

layer on the negative electrode increased over time. No material was observed to deposit on the positive electrode under the conditions studied. An additional control was an electrode in which no voltage was applied (designated as "neutral" electrode). As shown in Figure 3a, no deposition was observed on the surface of this "neutral" electrode. Figure 3b shows that when the concentration of chitosan in the solution was increased, there was increased deposition on the surface of the negative electrode. Again no deposition was observed on the positive electrode or on the control electrode in which no voltage was applied. Figure 3c shows that deposition on the negative electrode also increased with increasing voltage.

In summary, Figures 1 through 3 demonstrate that an applied voltage can be used to deposit a thin layer onto a negative electrode when the electrode is immersed in a chitosan solution. Additionally, the thickness of the deposited layer can be controlled by the deposition conditions. Finally, once the deposited layer is neutralized, it can be retained on the electrode surface even in the absence of an applied voltage (i.e. the electrode can be extensively rinsed). This latter observation is consistent with the fact that chitosan is insoluble under neutral and basic conditions.

We used two independent techniques to provide chemical evidence that the material deposited on the negative electrode is chitosan. For IR analysis, we recovered the "neutralized" material from the electrode surface, rinsed it extensively, dried it overnight, and incorporated the material into a KBr pellet. Figure 4 compares the IR spectrum for the KBr pellet of the deposited material with the spectrum of a chitosan film. The absorption spectra are similar for the two samples providing evidence that the material deposited on the negative electrode is chitosan. Some differences in the spectra are observed in the amine and amide regions (1500-

1700 cm^{-1})[28-30] suggesting the possibility that chitosan chains that are more highly deacetylated (and therefore more highly charged) may be preferentially deposited onto the negative electrode.

The second technique to provide chemical evidence that the deposited material is chitosan was provided by electrospray mass spectrometry (ES-MS). Because chitosan's molecular weight (> 300,000 g/mol) exceeds the limit for analysis, we enzymatically hydrolyzed the deposited material and analyzed the hydrolysate. For this analysis, the deposited layer was dissolved from the electrode surface into an acidic solution. After dilution, the solution was incubated with the chitosan-hydrolyzing enzyme, chitosanase.[31] Figure 5 shows the ES-MS results for this hydrolyzate.

To examine the results in Figure 5, it is necessary to consider the peaks expected for the enzymatic hydrolysis of chitosan.[27] Enzymatic hydrolysis of chitosan is known to result in the formation of various species (monomers, dimers, etc.).[32] Additionally, chitosan is a copolymer of glucosamine and N-acetylglucosamine, and the predominant oligomeric species are expected to consist of either glucosamine units or both glucosamine and N-acetylglucosamine units. Because the degree of acetylation is low (15 %), we do not expect significant amounts of oligomers that contain more than a single N-acetylglucosamine residue. Finally, it is known that MS spectra of glucosamine and glucosamine trimers contain product ions resulting from the loss of H_2O.[33] Table 1 lists a series of peaks expected for the hydrolysis of chitosan (e.g. various monomers, dimers, trimers, tetramers, and pentamers). By comparison of these expectations with results in Figure 5 (listed in parenthesis in Table 1), it is clear that the ES-MS provides strong evidence that the deposited material is chitosan.

A control in the ES-MS study was provided by a sample that was incubated in the absence of chitosanase. The ES-MS analysis of this control showed weak signals with a low signal-to-noise ratio (not shown). This is consistent with the expectation that un-hydrolyzed chitosan will be too large (300,000 g/mol) to be measured by ES-MS. The highest signals in this control appeared at *m/z* of 220 and 299 and the latter signal does not even appear in Figure 5. Thus, chitosanase-catalyzed hydrolysis of the deposited material was necessary to attain strong signals in the ES-MS.

Table 1. Expected and observed *m/z* values for enzymatically hydrolyzed chitosan. (Observed values from Figure 5 appear in parenthesis)

	Monomer	Dimer	Trimer	Tetramer	Pentamer
$(Gln)_x - 3H_2O$	126 (126)	287 (288)	448 (448)	609 (609)	770 (769)
$(Gln)_x - 2H_2O$	144 (144)	305 (306)	466 (467)	627 (625)	788 (789)
$(Gln)_x - H_2O$	162 (162)	323 (324)	484 (484)	645 (644)	806 (805)
$(Gln)_x$	180 (180)	341 (342)	502 (503)	663 (663)	824 (821)
$[GlcNAc \bullet (Gln)_{x-1}] - H_2O$	204 (205)	365 (364)	526 (525)	687 (686)	848 (847)
$[GlcNAc \bullet (Gln)_{x-1}]$	222 -	383	544 (545)	705 (705)	866 (864)

Gln: Glucosamine; GlcNAc: N-Acetylglucosamine.

In summary, two independent techniques were used to provide chemical evidence that the deposited material was chitosan. Standard IR analysis shows that the spectrum for the deposited material is similar to the spectrum for chitosan. Further, the deposited material was susceptible

to hydrolysis by the enzyme chitosanase while the hydrolysate shows a family of peaks consistent with glucosamine and N-acetylglucosamine residues – the repeating units of chitosan.

CONCLUSIONS

Chitosan is a unique biopolymer that we believe offers interesting possibilities for controlling the surface chemistry of devices. First, chitosan is an amine-rich polysaccharide that is positively charged under mildly acidic conditions. This characteristic allows a thin chitosan layer to be deposited (i.e. "self-assembled") onto a negative electrode in response to an applied voltage. The results reported here demonstrate that the thickness of the deposited layer can be controlled by the conditions used. Second, chitosan's pKa is rather low (pKa $\approx$ 6.3) compared to other amine-containing biopolymers (e.g. polylysine's pKa is 10.5), and above it's pKa chitosan is insoluble. As a result of this pH-dependent solubility, a simple neutralization step is sufficient to convert chitosan to an insoluble form that can be retained on the surface of the electrode (i.e. the applied voltage is only required for deposition and not to retain the chitosan layer). Third, the high content of primary amine groups allows a chitosan coating to be used for controlling surface properties and for subsequent modification steps. The utility of amine groups is illustrated by the current interest in creating amine-terminated monolayers.[2,34-38] The amine groups also enable biologically active molecules (e.g. peptides and proteins) to be coupled onto chitosan surfaces using standard coupling chemistries (e.g. glutaraldehyde- or carbodiimide-based chemistries)[39-42] or using enzymatic methods.[43,44] Finally, chitosan is gaining increasing attention as a biomaterial for applications ranging from enzyme immobilization[45-49] to the

creation of biocompatible surfaces.[50-53] Thus, chitosan may provide an appropriate interface between biological systems and microelectronic devices.

The prior example is provided as illustration of the disclosed invention and is not intended to limit the scope of the invention. All cited references are herein incorporated in their entirety by reference.

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Claims

We claim:

1. A method of depositing chitosan onto a metal substrate comprising the steps of :

    a) contacting said substrate with a chitosan solution; and

    b) applying an electric current to the substrate, wherein the substrate is in a circuit with an electrode also in contact with said chitosan solution.

2. The method of claim 1, further comprising washing the substrate containing deposited chitosan with at least one liquid selected from the group consisting of water, a solution with neutral pH, a basic solution and an acidic solution.

3. The method of claim 1, further comprising contacting the substrate with bound chitosan with chitosanase.

4. A substrate coated with chitosan.

5. A substrate coated with chitosan wherein the substrate is a metal.

6. The substrate of claim 4, further comprising bound protein.

7. The substrate of claim 6, wherein the bound protein is an enzyme

8. The substrate of claim 4, further comprising bound polynucleotide.

9. The substrate of claim 8, wherein the polynucleotide is RNA

10. The substrate of claim 8, wherein the polynucleotide is DNA.

11. The substrate of claim 4, further comprising bound cells.

Abstract

Disclosed is the assembly of the amine-rich polysaccharide - chitosan – from solution onto electrode surfaces as a result of voltage bias on the electrode. Chitosan is positively charged and water-soluble under mildly acidic conditions, and is uncharged and insoluble under basic conditions. Chitosan is deposited from acidic solution onto the surface of a negative electrode and the thickness of the deposited layer is on the order of a micron. The thickness of the deposited layer is dependent upon the deposition time, the applied voltage, and the chitosan concentration. Once deposited and neutralized, the chitosan layer can be retained on the electrode surface without the need for an applied voltage. Infrared (FTIR) and electrospray mass spectrometry (ES-MS) confirmed that the deposited material was chitosan. Chitosan can be deposited and retained on electrode surfaces and advantages for applications in microfabricated devices are disclosed.

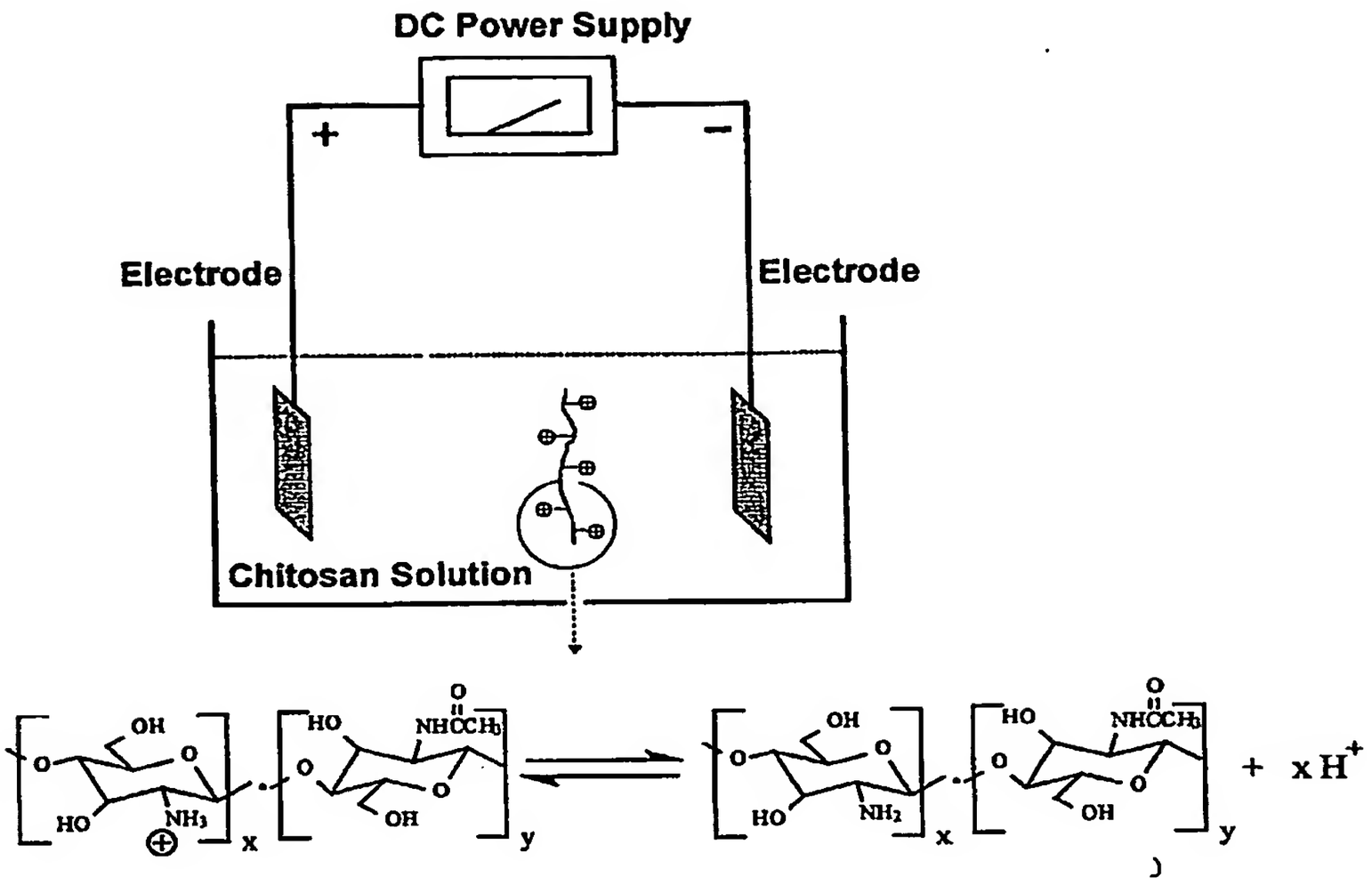


Figure 1

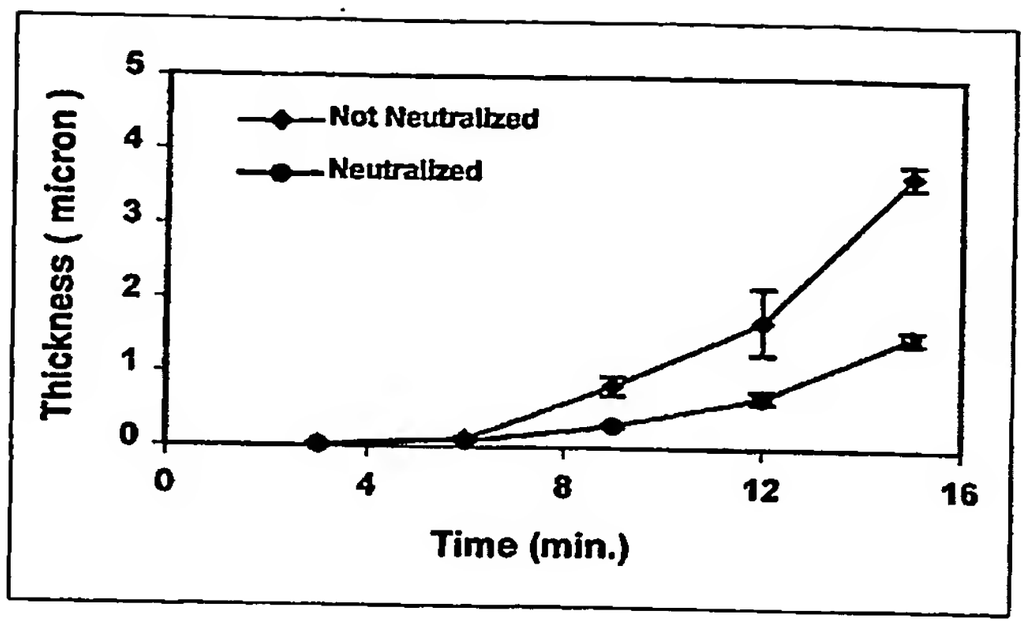
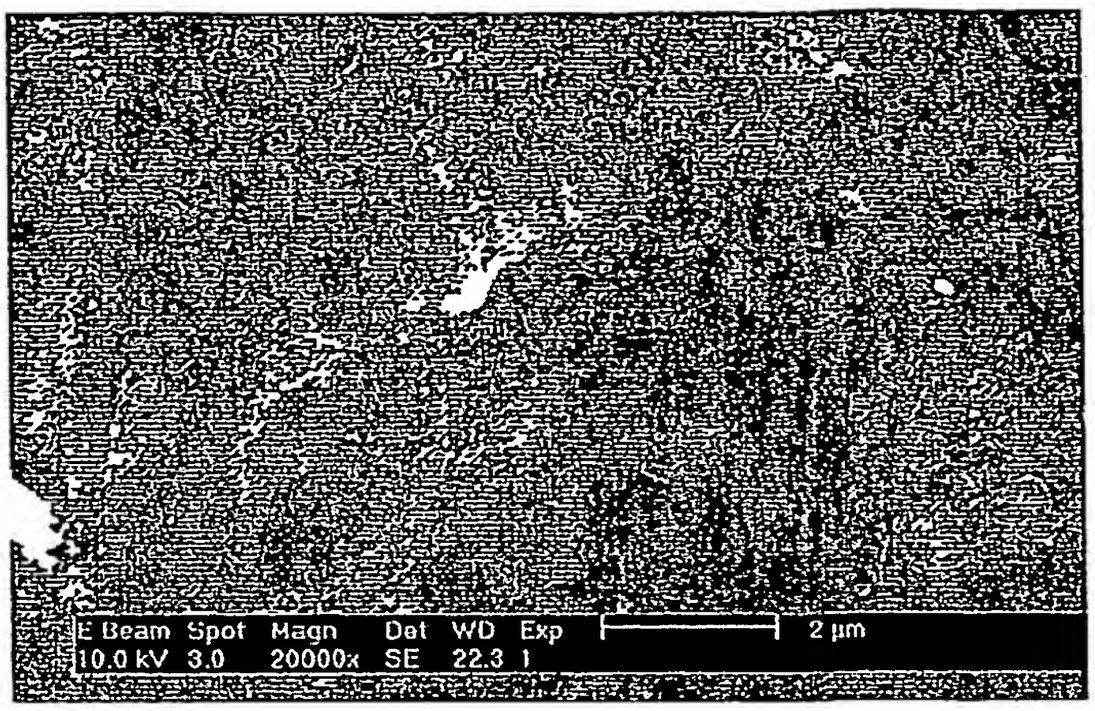
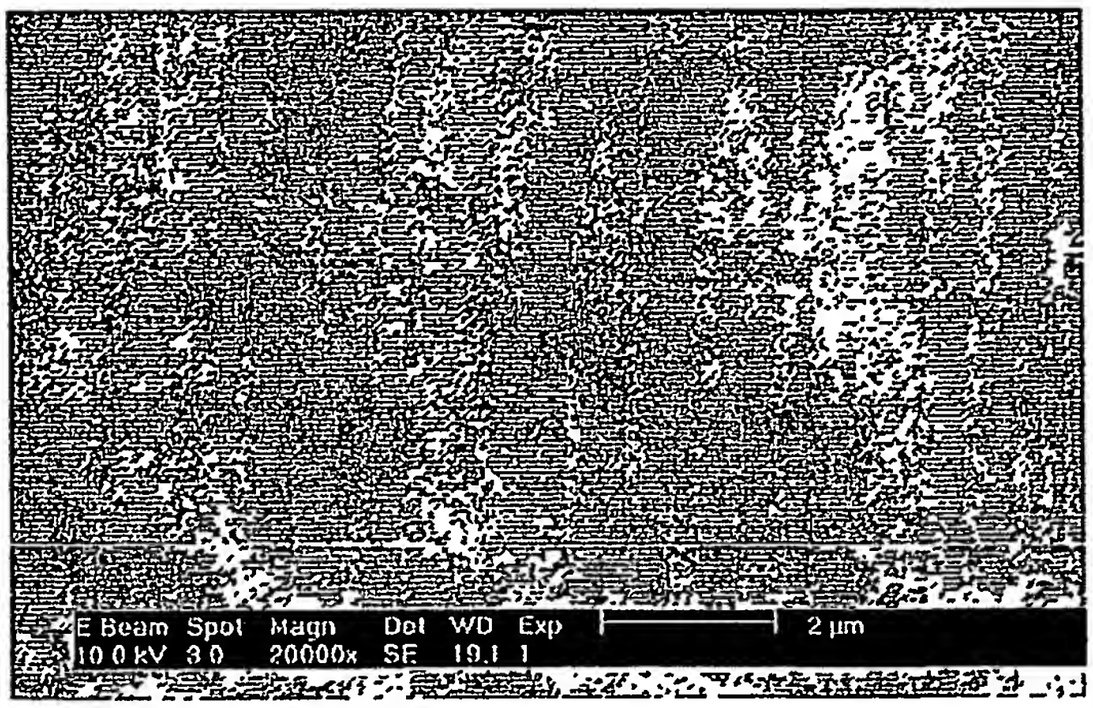

Thickness (micron)
Not Neutralized
Neutralized
0
1
2
3
4
5
0
4
8
12
16
Time (min.)

Figure 2

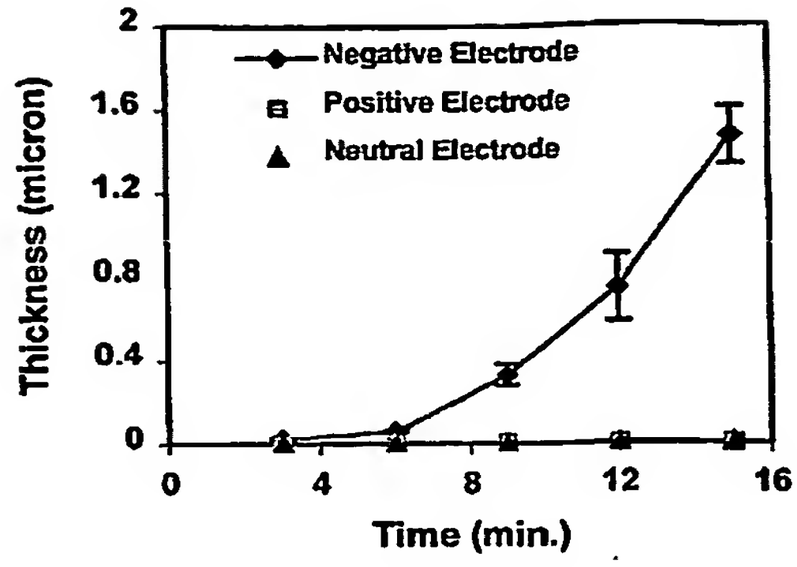

E Beam Spot Magn Det WD Exp
10.0 kV 3.0 20000x SE 22.3 1
2 µm

(a)

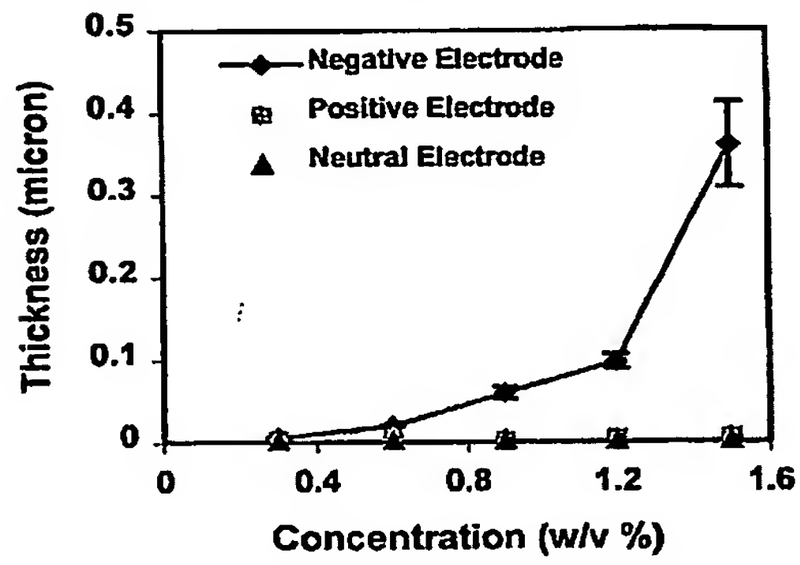

E Beam Spot Magn Det WD Exp
10.0 kV 3.0 20000x SE 19.1 1
2 µm

(b)

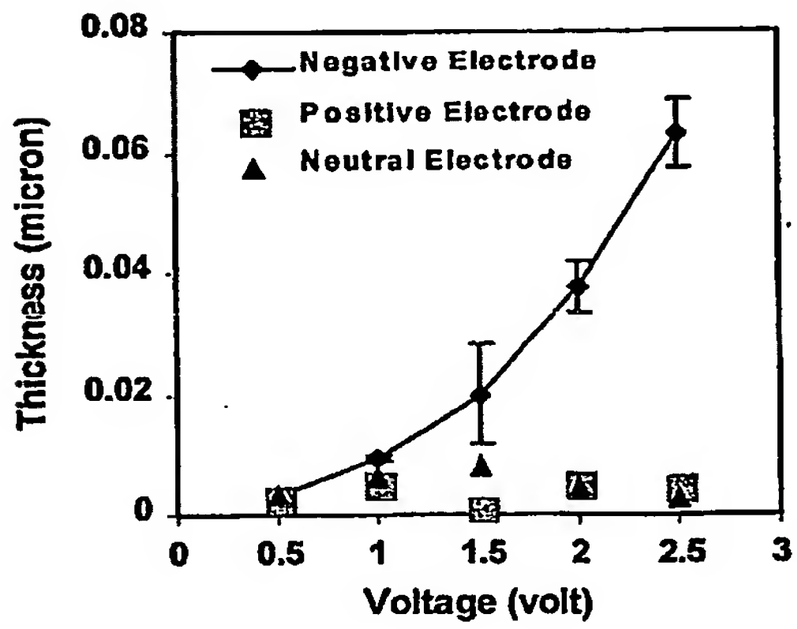
Figure 3


Negative Electrode
Positive Electrode
Neutral Electrode
Thickness (micron)
Time (min.)
0
0.4
0.8
1.2
1.6
2
0
4
8
12
16

(a)


Negative Electrode
Positive Electrode
Neutral Electrode
Thickness (micron)
Concentration (w/v %)
0
0.1
0.2
0.3
0.4
0.5
0
0.4
0.8
1.2
1.6

(b)


Negative Electrode
Positive Electrode
Neutral Electrode
Thickness (micron)
Voltage (volt)
0
0.02
0.04
0.06
0.08
0
0.5
1
1.5
2
2.5
3

(c)

Figure 4

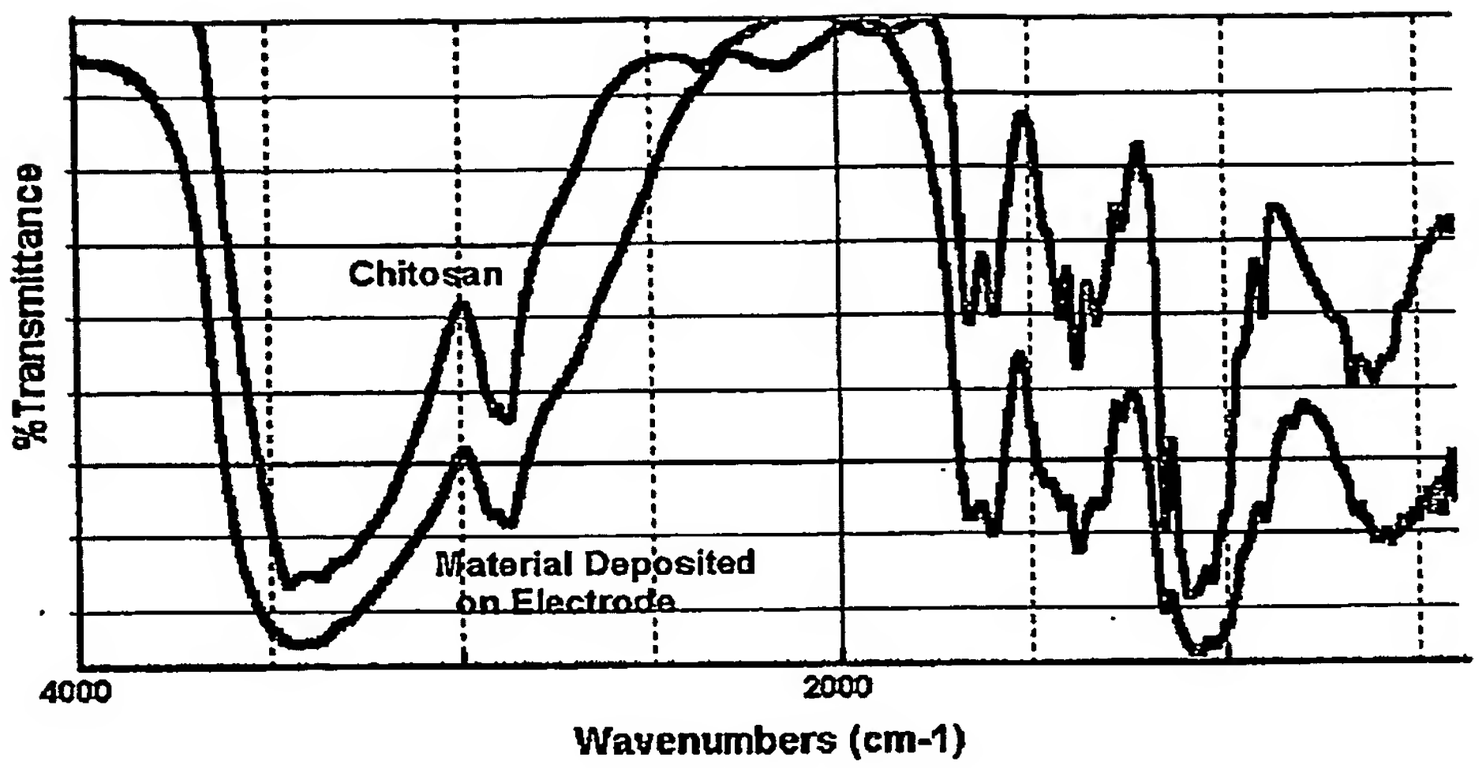

%Transmittance
Chitosan
Material Deposited
on Electrode
4000
2000
Wavenumbers (cm-1)

Figure 5

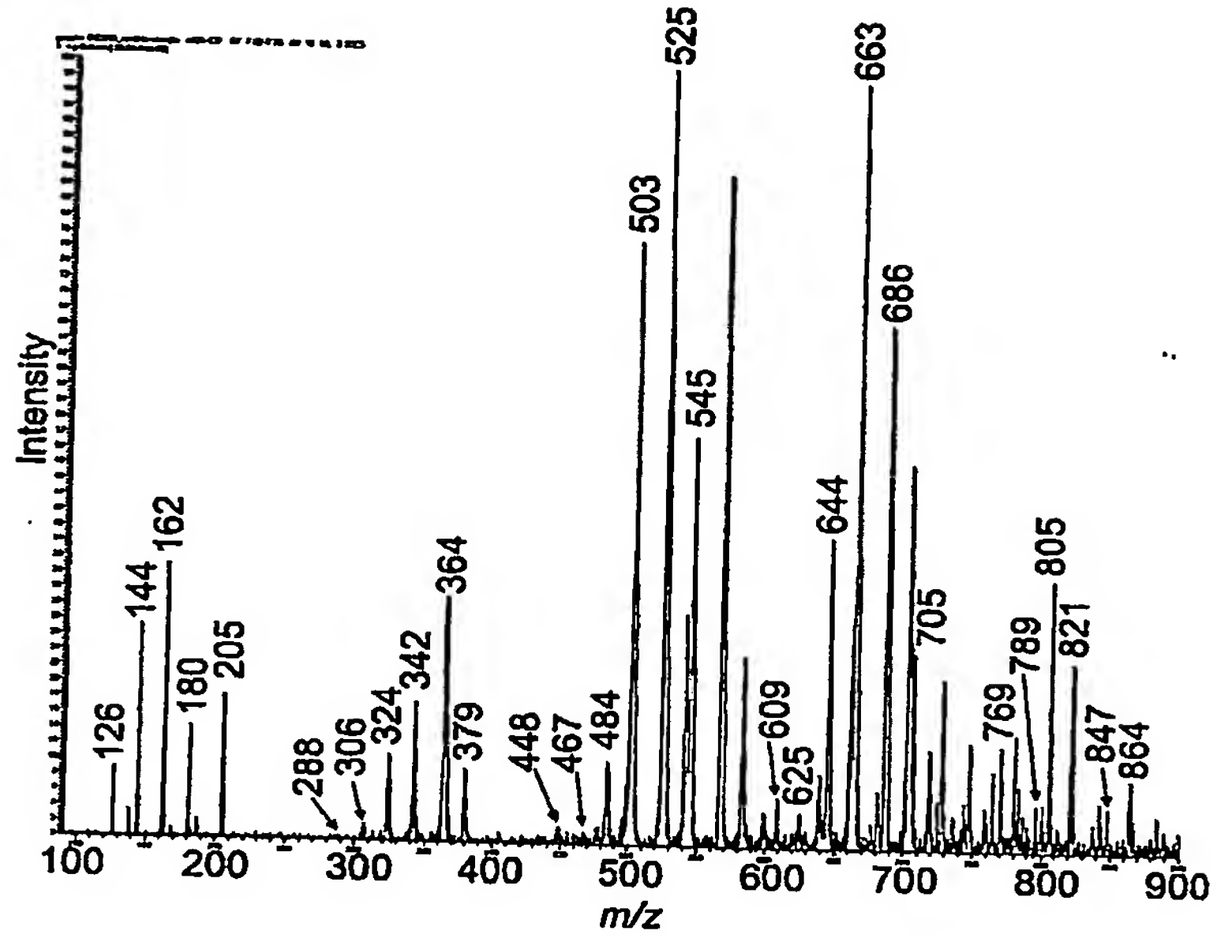

Intensity
126
144
162
180
205
288
306
324
342
364
379
448
467
484
503
525
545
609
625
644
663
686
705
769
789
805
821
847
864
100
200
300
400
500
600
700
800
900
m/z

Figure 6

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